REVIEWS

Alternative Methods for Eye and Skin Irritation Tests: An Overview

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ABSTRACT: The evaluation of eye and skin irritation potential is essential to ensuring the safety of individuals in contact with a wide variety of substances designed for industrial, pharmaceutical or cosmetic use. The Draize rabbit eye and skin irritancy tests have been used for 60 years to attempt to predict the human ocular and dermal irritation of such products. The Draize test has been the standard for ocular and dermal safety assessments for decades. However, several aspects of the test have been criticised. These include: the subjectivity of the method; the overestimation of human responses; and the method's cruelty. The inadequacies of the Draize test have led to several laboratories over the last 20 years making efforts to develop *in vitro* assays to replace it. Protocols that use different types of cell cultures and other methods have been devised to study eye and skin irritation. Different commercial kits have also been developed to study eye and skin irritation, based on the action of chemicals on these tissues. This article presents a review of the main alternatives developed to replace the use of animals in the study of chemical irritation. Particular attention is paid to the reproducibility of each method. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:46–59, 2008

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EYE AND SKIN IRRITATION

The main goal of toxicological scientific endeavours is to safeguard human beings against the possible adverse effects of diverse types of chemicals, including pharmaceuticals, cosmetics, household products, industrial chemicals, and agrochemicals. The exposure can be incidental, accidental, or intentional, as with cosmetics and certain drugs. One of the possible effects of the exposition and accidental contact with new chemicals is eye and skin irritation. In general, the physiological response to a chemical stimulus is irritation, which involves objective changes (e.g., local redness and oedema) and subjective sensations (e.g., pruritus and pain).

Before humans can be exposed to such substances, the tendency of new chemicals to cause eye and skin irritation must be determined. Assessment of eye and skin irritation potential is an important part of any comprehensive toxicology programme for new chemicals and consumer products. Even today, the final preclinical safety assessment of chemicals is largely based on animal experiments. However, ethical concerns involving the use of laboratory animals, the validity of animal eye and skin as human eye and skin models, and the need for more efficient and

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cost-effective preclinical validation methods have promoted the development of alternative methods to assess irritation.

THE DRAIZE EYE IRRITATION TEST

Ocular toxicology developed during the 20th century as the pharmaceutical industry grew and some drugs were found to have effects on the eye.[1] The main objective of research was to find methods that could test the potential irritation of new products. This research led to a system for quantitatively assessing the toxicity of topical compounds. In their first article, Draize and his colleagues proposed an approach for cutaneous and ocular testing and described how to assess acute, intermediate and chronic exposure by applying compounds to the skin, penis, and eyes of rabbits.[2]

Following this initial report, Draize's techniques were used by the Food and Drug Administration (FDA) to evaluate the safety of several substances, such as: insecticides, sunscreens and antiseptics.[3] The method was adopted by other laboratories for screening many compounds. Scientists began to refer to this technique as the 'Draize technique' or the 'Draize test'.[4]

The Draize test assigned values and weights to different parameters to represent their contribution to the overall picture. In the eye irritation test, the cornea and iris were heavily weighted because of their vital role in vision.[5] A method based on that described by Draize and coworkers became the authoritative testing process. In 1964, it was incorporated into FDA regulation for a short period.

The Draize eye test involves several phases. Each of these phases has come under scrutiny. The main areas that have been questioned are: the test performance; the test reproducibility and relevance; the use and interpretation of test scores. The test was developed to evaluate products that are designed to come into contact with the eye and the area surrounding the eye, such as ophthalmological preparations and cosmetics. For other products, such as household and industrial chemicals, the purpose of risk assessment is to provide handling guidelines and label precautions. The method is based on applying the test substance to the rabbit eye and evaluating the damage caused to the cornea, iris, and conjunctiva. Ocular responses are scored at 1, 24, 48, and 72 h and at 7 days. The exposure of the ocular surface to a foreign material may result in a response ranging from slight redness to severe injury with loss of the corneal epithelium, damage to the underlying stroma and loss of vision. However, instillation of a fixed amount of a compound onto the rabbit eye is not necessarily consistent with human accidental exposure. Draize's article became one of the most cited publications in toxicology. Nevertheless, by 1960, the eye and skin test was coming under criticism. The high number of variables in the Draize test limits its reproducibility,[6] especially for moderately irritating compounds. Animals of the same species and strain can respond in various ways to a given compound, and different examiners can obtain diverse results from the same test.[7,8] The anatomy and biochemistry of the rabbit eye is different to that of the human eye. Physiological reasons why the rabbit eye may not adequately predict effects include: rabbits' relatively low tear production, blink frequency, and ocular surface sensitivity.[9]

Revisions have been incorporated into the procedures for Draize testing. Modifications aim to derive the maximum information from the fewest animals, while retaining a good correlation with expected human reactions.

Another way to avoid testing irritating products on the eye is to assess skin irritation before proceeding with ocular instillation.

A possible alternative to the classical method for assessing eye irritation potential is the Low Volume Eye Test (LVET).[10,11] This method gave a better correlation with human responses than the Draize test. The human exposure data used in the study were: reported consumer eye incidents with detergents, soap, shampoo, and other household products,[12] and reports of eye accidents that employees had during manufacture. Both the LVET and the Draize test overestimate the human response to accidental eye exposure. However, the LVET is better at assessing eye irritation than the Draize test and has been proved to be an acceptable alternative.[13]

THE DRAIZE SKIN IRRITATION TEST

Acute skin irritation is evaluated *in vivo* in rabbits after they have been shaved. The product is applied to the skin and the appearance of oedema and/or erythema is evaluated at 1, 24, 48, and 72 h after application. The scoring system enables products to be classified from nonirritant to very irritant.

A number of *in vivo* models exist as alternatives to the Draize test. Some of these use other species such as: the guinea pig, mouse and rat.[14] Alternative parameters to erythema and oedema have also been developed. These include: cutaneous blood flow, as measured by Laser Doppler Flowmetry, infrared detection of skin temperature, and skin thickness assessment. Alternatives to the Draize occluded and semi-occluded patch systems include using an open application. Other authors suggest that, if attention is paid to ethical considerations, skin irritation tests could be carried out on humans. As rabbit skin is much more reactive than human skin, some authors state that rabbit responses cannot accurately be used to predict human responses.[13]

VALIDATION OF ALTERNATIVE METHODS

If alternative methods are to be successfully incorporated into the safety assessment process, it will be necessary to demonstrate that the new procedures can provide at least an equivalent level of protection to that obtained with current methods. Additionally, if deadlines imposed by legislation such as the 6th Amendment to the European Union Cosmetics Directive are to be met, it is important that the validation process be conducted in a manner that efficiently and definitively characterises the performance of the alternative methods.[15] Validation has been defined as 'the establishment of the reliability and relevance of an alternative method for a specific purpose'. To assess the validity of an alternative method, it is important to clearly define the terms reliability and relevance. For a toxicologist to rely on an alternative method, two things must be known about its performance. First, it must be possible to consistently reproduce the results from an alternative method. Second, it must be possible to consistently and correctly convert the results from the alternative method into useful predictions of toxicity so that appropriate safety assessments can be made. Thus, reliability may be defined as the establishment of the reproducibility of the data obtained from a method across different laboratories and the reproducibility of the predictions of toxic hazard after application of a clearly stated prediction model to the alternative method data across appropriately defined sets of test substances. Once the reproducibility of an alternative method has been confirmed, then its relevance must be evaluated. Relevance has been defined as establishing the scientific meaningfulness and usefulness of results from an alternative method for a particular purpose. Establishing usefulness and meaningfulness is important because hazard predictions obtained from scientifically credible alternative methods have a higher probability of being correct. To establish relevance, all available information related to the fundamental scientific basis, reliability (as defined above), and practical operation of the alternative method, and to the *in vivo* toxicity test to be replaced must be thoroughly reviewed. Ultimately, a judgment must be made about whether or not a method is relevant for a particular purpose (Tab. 1).

Validation studies that have been conducted to date can be classified on the basis of their apparent objectives. These include: in-house validation, validation for commercial purposes, and validation which is undertaken to try to secure regulatory acceptance of a new test. Regardless of the purpose or the type of validation study, it must be of a scientific rather than a political nature.[16]

Any new method that is considered to have been adequately validated as a replacement for an existing method has to receive as widespread international recognition as possible. The OECD has established a procedure for updating test guidelines and for introducing new test methods.[17] This takes into account both scientific advances and proposals based on animal welfare considerations.The European Centre for the Validation of Alternative Methods (ECVAM) and The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) were created in Europe and the United States respectively, in order to develop alternative methods. ECVAM's mission is to promote the scientific and regulatory acceptance of nonanimal tests that are of importance to the biomedical sciences. This mission is carried out through research, test development, validation, and the establishment of a specialised database service. To coordinate at the European level, the relevance and reliability of tests for specific purposes need to be independently evaluated. Thus, chemicals and different kinds of products, including medicines, vaccines, medical devices, cosmetics, household products, and agricultural products, can be manufactured, transported and used more economically and more safely. Simultaneously, the current reliance on animal test procedures can be progressively reduced.[16,18]

Table 1. *In Vitro* Alternative Methods to Evaluate Irritation *r*

In Vitro Alternatives	Endpoints	References
In vitro eye irritation tests		
Red blood cell test	Haemolysis and haemoglobin denaturation	27–32
Haemoglobin denaturation	Spectrophotometric changes in haemoglobin	33–37
Chorioallantoic membrane	Haemorrhage, vasoconstriction, coagulation, trypan blue adsorption	38–52
Isolated cornea	Corneal opacity	53–54
Isolated eyes	Corneal swelling, corneal opacity, fluorescein retention	55–56
Cell culture	LDH, MTT, fluorescein leakage, neutral red release	57–65
Commercial kits		
Eytex	Cloudy	66–67
Reconstituted corneal epithelium	Cell death	68–69
In vitro skin irritation		
Cell culture	Neutral red uptake, IL-1α	70–81
Commercial kits		
Skintex	Turbidity	82
Human epidermal models	Cell death, IL-1α	83
In vitro skin corrosivity		
Commercial kits		
Corrositex	Colour change	84
Epiderm	MTT	85–86
Episkin	MTT	87–92
TER	Reduction in transcutaneous electrical resistance	93, 94
In vitro phototoxicity		
Red blood cell phototoxicity	Photohaemolysis	96
3T3 NRU phototoxicity	Neutral red uptake	97–99
Keratinocyte cell culture	MTT	62, 100–102
Skin equivalent model	Cell death	103

ICCVAM's mission is to facilitate the development, validation and regulatory acceptance of new and revised regulatory test methods that reduce, refine and replace the use of animals in testing, while maintaining and promoting scientific quality and the protection of human health, animal health, and the environment. In Japan, the Japanese Society of Alternatives to Animals Experiments (JSAAE) is responsible for carrying out validation assays. In recent years, different studies have been developed to validate alternatives to the eye[19–23] and skin[24,25] irritation *in vivo* test. However, despite the quantity and quality of the work carried out, few alternative methods to replace the classical Draize test have been accepted by the regulatory authorities.

ALTERNATIVES TO THE EYE IRRITATION TEST

The ocular surface is a complex system consisting of corneal and conjunctival epithelial cells, the underlying stroma, and associated cells. Its complexity makes it difficult to develop alternatives tests with a physiological and mechanistic base that are capable of eliminating the need for animals.[26]

The following different methods have been proposed as alternatives.

Red Blood Cells Test

Red blood cells (RBCs), which are readily available, have a long scientific history of being used in the study of the lysis of plasma membranes. The RBC test was developed to assess initial cellular reactions to the irritation caused by certain chemicals.[27–29] Certain classes of chemical irritants damage cell plasma membranes and denature several types of proteins. Nonirritant surfactants will not cause these reactions. It is hypothesised that such reactions can be correlated with the initial events in eye tissue irritation, leading to inflammatory responses of the tissue and changes in protein conformation. Such events occur, for instance, in the opacification of the cornea after contact with chemicals. The method

is based on measuring the haemolysis induced in erythrocytes, and the haemoglobin denaturation (HD). The L/D ratio can be calculated from the haemolysis concentration and the HD. This value enables compounds to be classified as a function of their potential ocular irritation. Results can then be correlated with *in vivo* data.

The RBC test is a validated alternative to the Draize eye irritation test for the acute effects of typical surfactant-based formulations and ingredients.[30] The mechanism of the RBC test is clear and the method is simple. It does not require any special techniques or equipment. This method also has the merit of being rapid and inexpensive. It correlates well with the *in vivo* method,[31] and is particularly valid for the study of surfactants[32] and cleaning products containing surfactants.

The Haemoglobin Denaturation Test

This has been proposed as a method for predicting eye irritation by measuring spectrophotometric changes in haemoglobin.[33] It is based on the hypothesis that the eye irritation produced by surfactants is a consequence of protein denaturation. The original method was based on calculating the haemoglobin denaturation ratio (HDR) from the absorbance results and then expressing them as a percentage. The authors proposed a formula that correlated with the Draize total score or with the Draize corneal score.[34] Due to the complexity of the formula, we have proposed a modification of the method. This is based on plotting the results of HDR versus the log concentration of the product in W/V or V/V for solids and liquids, respectively.[35]

Other modifications of the method have evaluated HD by measuring both the optical density at 418 nm and the maximum absorption wavelength. The HD was then compared with that of a positive control and expressed according to three indices: the test substance concentration that induces 50% of the HD induced by the positive control; the relative HD rate induced by 1% of the test substance; and the change in the maximal absorption wavelength caused by 1% of the test substance.[36] This method should not be applied to coloured materials if the absorption of the material is strong at around 418 nm. However, the method appears to correlate well with corneal opacity and may be a good predictor of strong eye irritation.

This method is easy to handle. Test substances are dissolved and serially diluted with a standard buffer solution on a 96-well microplate. The haemoglobin solution is then added. After 5 min incubation, the optical density is measured using a multiplate reader. This method could be used as an initial screen in a system that includes other assays for evaluating mechanisms other than protein denaturation. The test does not require aseptic handling, and the results can be obtained quickly. Thus, an initial screen using this test would certainly reduce the time required for *in vitro* risk assessment. Furthermore, a combination of the HD test and the RBC lysis test correlates highly with the total Draize score and the Draize test results.[37]

Chorioallantoic Membrane

The chorioallantoic membrane (CAM) is the vascularised respiratory membrane that surrounds a chick developing inside an egg. This assay involves isolating an area of this membrane. A test material is applied to the prepared surface. After an incubation period that varies depending on the protocol used, the membrane is inspected visually and changes in its morphology are scored. There are several variations in CAM assay protocols.[38–40] One of the scores determines the irritation potential from a formula which includes the time in seconds at which haemorrhage, vasoconstriction and coagulation appears. The irritation potential ranges from 0 to 21. Substances are categorised according to these values.

This method allows different type of substances, such as surfactants,[41] cosmetics, ingredients,[42] different chemical products,[43–45] or dental adhesives[46] to be evaluated. In the case of cosmetic formulations, the results mainly correlate with the presence and concentration of surfactants in the test article.[42]

The Cosmetic Toiletry and Fragrance Association performed an evaluation of alternatives to the Draize primary eye irritation test. They concluded that none of the assays evaluated exhibited 100% sensitivity and 100% specificity. However, the CAM method achieved 94% sensitivity and 100% specificity.[47]

The combination of histological and visual HET-CAM tests is of interest, as it may give a more sensitive evaluation of the innocuousness of cosmetic active ingredients.[48]

A modification has been introduced to reduce the subjectivity of the method. This involves using trypan blue stain. The amount of stain fixed on the damaged membrane is quantified.[49,50] This method has the advantage of being able to evaluate dye substances.[51] We have introduced a modification to the method, whereby the membrane weight is measured after dissection and the results are expressed as a function of the membrane weight.[52]

Isolated Cornea Opacity and Permeability Test

In this procedure, bovine eyes are collected from a slaughterhouse and carefully examined. Any eyes that present a defect are discarded. Selected cornea are quickly mounted onto specially designed holders[53] composed of two separate chambers. Both chambers are then filled with Eagle minimum essential medium (EMEM), supplemented with 1% foetal calf serum. Corneas are incubated for 1 h at 32°C. Fresh medium is then added to the posterior compartment (endothelial side). The anterior compartment (epithelium side) receives either the test compound or its vehicle. Three corneas are treated with the test compound and three with the vehicle. At the end of the exposure time (10 min), the epithelial side is washed and the anterior compartment is refilled with medium. After 2 h of incubation, the opacity of each cornea is measure with an opacimeter. Once the opacity reading has been completed, the medium is removed from both compartments of the holders. Fresh medium is added to the posterior compartment and a Na-fluorescein solution is added to the anterior compartment. Corneas are then incubated horizontally for 90 min. The amount of dye that passes through the cornea is measured spectrophotometrically at 490 nm and expressed as an optical density value. The means of both endpoints (opacity and optical density) are calculated for each test sample. A score is then established by adding the opacity value and 15 times the optical density value.[54] This calculation is performed for each product.

Isolated Eyes

The enucleated eye test (EET) has been recognised as a valuable alternative to the Draize test. This test uses the isolated eyes of rabbits. It is the test system that is closest to the *in vivo* test, without needing to use live animals. Three parameters are measured in this ex vivo bioassay. These are: corneal swelling, corneal opacity, and fluorescein retention. The measurement of corneal swelling in this assay is a highly objective, and discriminative parameter. In combination with the detailed observation of corneal opacity and fluorescein retention, a reliable evaluation of the eye irritation potential of test materials is achieved.[55]

On the basis of the data submitted about each test, it was concluded that the isolated rabbit eye test, as performed, was capable of screening for severe eye irritants. However, it was of no practical value for determining the full range of irritation potential. The isolated chicken eye (ICE) test, as performed, showed promise as a method for predicting eye irritation potential. However, the database is too small and needs to be expanded. The bovine corneal opacity test has an extensive database and performed reasonably overall at screening out severe irritants. It also performed well at assigning relative potencies. The bovine lens test should be researched further in order to demonstrate its utility.[56]

The ICE is an organotypic model that provides short-term (4 h) maintenance of the whole eye. The ICE was developed as a modification of the isolated rabbit eye test method. It was intended as a screening assay to identify the ocular corrosiveness and severe irritation potential of products, product components, individual chemicals, or substances. The ICE test method may also be useful as one of several tests in a battery of *in vitro* eye irritation methods that collectively predict the eye irritation potential of a substance *in vivo*.

Cell Culture

Different types of cells have been cultured and different endpoints determined to assay eye irritation potential. The test is carried out by measuring membrane integrity, cell viability, and growth.[26] Primary cultures of rabbit corneal epithelial cells have been used in such assays. Lactate dehydrogenase enzyme leakage (LDH) and MTT mitochondrial reduction are measured.[57]

Other cells that have been used are cultured human corneal endothelial cells and human retinal pigment epithelial cells.[58]

Another study proposes a three-dimensional model of bovine corneal stroma and epithelium that is not only easy to reproduce but could also be used in the toxicological field as an alternative to animal

experimentation. Using this model, epithelium with similar characteristics to those of *in vivo* epithelium is grown. Basal cells are cube-shaped, whereas superficial areas are horizontally longer; desmosomes and 64 kDa keratin (a marker for differentiating corneal epithelial cells) are both expressed and the basal lamina is synthesised.[59]

The fluorescein leakage test is based on the principle that corneal epithelium can function as an impermeable barrier to potentially hazardous chemical substances. The corneal epithelium is mimicked *in vitro* by adherently growing epithelial cells. The test is usually performed using MDCK cells, NHEK, or human corneal cells.[60–62]

In the silicon microphysiometer assay, chemical substances induce changes in the metabolic activity of adherent cell cultures of NHEK or L929 lines. These changes serve as measure of irritation potential.[63,64]

One report discusses the regulatory experience gained over the last 20 years with the EU chemicals notification procedure, with respect to the assessment of eye lesions observed in Draize tests. The aim of this report is to promote the development of specific *in vitro* assays which can discriminate between eye damage, moderate eye irritation and minor irritation effects that are completely reversible within a few days.[65]

Commercial Kits

The EYTEX™ system is an assay for eye irritation which uses a vegetable protein extracted from jack beans. Like the cornea of the eye, this clear protein gel becomes cloudy when it comes into contact with an irritant. The molecules in the Eytex protein gel also have a highly organised structure which is changed by irritants. The more irritant a substance is, the more the structure of the molecule groups is changed and the cloudier the gel appears. Results of the Eytex test correlate well with Draize test results. However, ideally the Eytex test should be compared to information about human eye irritation, since the Draize test itself is not accurate. Eytex is better at identifying irritants than at identifying nonirritants.[63] An overall concordance of 80% was found between the EYTEX™ results and the *in vivo* assay. The assay error was 20%. Of this, 18% was due to an overestimation of the sample's irritation potential (false positives) and 2% was attributed to underestimation (false negatives). The EYTEX™ system protocols, when used appropriately, can provide a conservative means of assessing the irritation potential of most cosmetic formulations and their ingredients.[66,67]

Another promising method that is currently being reviewed by the Interagency ICCVAM is EpiOcular.™ Sponsored by the Colgate-Palmolive Company, EpiOcular™ is a three-dimensional, *in vitro* tissue model of the human corneal epithelium. The model consists of normal, human-derived epidermal keratinocytes cultured on a permeable polycarbonate membrane. This construct forms a stratified, squamous, multi-layered epithelium similar to that of the cornea. The tissue construct has an air–liquid interface and exhibits morphological and growth characteristics that are comparable to characteristics of the viable human eye. Chemicals can be directly applied to the surface of the tissue to predict potential eye damage in humans. The time to 50% cell death (ET50) is the endpoint for comparing different potential toxicants. The model provides data that reflect how cell cytotoxicity can be measured and related to the assessment of ocular irritation. This test appears to accurately predict the ocular irritation effects of chemicals and provides an *in vitro* alternative to the Draize test.[68,69]

ALTERNATIVES TO THE SKIN IRRITATION TEST

The effort to eliminate animal tests has also led to the development of a novel human patch test for assessing acute skin irritation potential. A case study shows the benefits of *in vitro* and human skin irritation tests compared to the animal tests they seek to replace. Strategies now exist to adequately assess human skin irritation potential without having to rely on animal test methods. Different *in vitro* alternatives have been proposed to replace rabbits in studies of skin irritation.[70]

Cell Culture

There are various ways of measuring damage when an irritant is applied to cell culture. For example, cells can be examined under the microscope, membrane damage can be assessed by enzyme leakage, or inflammation can be determined by the release of interleukins. Whatever method is used, the result can be measured

accurately. In contrast, in animal studies the results are measured subjectively by observers, who estimate the degree of swelling or redness.

One of the proposed alternative methods is the human skin keratinocyte cytotoxicity/neutral red assay. This is based on the ability of cultured human keratinocytes to take up the vital dye neutral red. Viable cells will incorporate the dye and damaged cells will not. Thus, the test system measures the response of human epidermis derived epithelial basal cells (keratinocytes) to test materials applied in culture. The concentration of test material that inhibits neutral red uptake by 50% (NR50) is calculated from dose inhibition curves. The NR50 values for different test materials are a measure of their *in vitro* cytotoxicity potential. This is then taken to be an indicator of the ultimate skin irritation potential.[71,72]

When activated by various irritants, keratinocytes are able to express or overexpress the production and/or release of inflammatory mediators, especially IL-1 type cytokine.[73–76] The importance of these reactions in the development of cutaneous irritation has led to assays of some inflammatory mediators that are produced *in vitro* by keratinocytes. Such assays are used as the first in a series of *in vitro* irritation tests.[76]

Other cells that can be used to study irritation are fibroblasts. Neutral red uptake in mouse embryo fibroblast 3T3 cells has been used for a long time as an endpoint.[77–79]

A three-dimensional model has also been used. This skin model has two layers, like the dermis and the epidermis of real skin and includes the cornified layer. Skin irritation in this model is measured by cell viability.[80]

Good laboratory practices need to be applied in cell culture procedures. Standard operation procedures have to be established for all the procedures developed during the test. In addition, special attention should be paid to training.[81]

Commercial Kits

The reconstructed human epidermis is a multilayered human skin grown in the laboratory. It can be used to test skin irritation. It is sold commercially under trade names such as Skin Squared™ and Episkin™. The Skintex dermal toxicity assay uses protein denaturation and changes in the conformation of other macromolecules as an endpoint on which the toxic potential of a compound is graded. The Skintex assay can best be described as a two-compartment, physicochemical model. The first compartment consists of a barrier membrane made up of a keratin/collagen matrix to which a red indicator dye is affixed. The compound of interest is placed directly onto the surface of this membrane. Subsequent interactions between the compound and the protein-aqueous material lead to variable degrees of denaturation, resulting in the release of the indicator dye. The compound then diffuses through the membrane and enters the second compartment. This compartment is described as a macromolecular matrix that consists of collagen, glycosaminoglycans, free fatty acids, amino acids, phospholipids, and buffer salts. Since these components are present in a highly ordered arrangement, this aqueous phase of the model has a baseline translucency. Interactions between the compound and the various components of this highly ordered matrix lead to further denaturation, resulting in the production of variable degrees of turbidity. The final result of a compound's interaction in the two compartments is therefore the sum of dye released from the first compartment and the turbidity produced in the second compartment. Both of these parameters are measured together spectrophotometrically. The final degree of perturbation is described as a Skintex Opacity Unit. The sensitivity of this method is higher than its specificity. Therefore, this method may tend to produce false positive errors.[82]

The efficacy of different human epidermis models in assessing the irritation of compounds has been reviewed recently.[83]

ALTERNATIVES TO SKIN CORROSIVITY

Commercial Kits

The CORROSITEX® assay is a standardised and quantitative *in vitro* corrosivity test. The potential corrosivity of a test material is assessed by measuring the time required for a chemical to 'breakthrough' a biobarrier membrane (reconstituted collagen matrix) and produce a colour change in a chemical detection system. The time at which a colour change is observed is recorded manually. The average breakthrough time of four replicates is used to determine whether a chemical is corrosive or not. Although this method has been accepted, the Corrositex assay accurately predicted a corrosive endpoint in only 57.1%

of chemical formulations identified as corrosive by *in vivo* evaluations. When compared with *in vivo* results, Corrositex correctly classified as corrosive or noncorrosive 37.5% of the formulations tested. A concordance of 20.8% was calculated for the packing group assignments of the evaluated formulations. The Corrositex assay did not accurately predict a corrosive endpoint or packing group assignment for all of the formulations used in this study. Manufacturers should assess this method's relevance to their products before using it to comply with hazardous material and worker safety regulations.[84] Corrositex is a validated and accepted dermal corrosion test method for classifying substances.

The EpiDerm™ skin model consists of normal, human-derived epidermal keratinocytes (NHEK) which have been cultured to form a multi-layered, highly differentiated model of the human epidermis. Chemicals can be applied directly to the culture surface, at the air interface. In this way, undiluted and/or end use dilutions can be tested directly. Ultrastructurally, the Epiderm model is closely parallel to human skin. Therefore, it provides a useful *in vitro* way of assessing dermal irritation and corrosivity. This test can be used as a prescreen to assess the dermal irritation potential prior to conducting an *in vivo* test. The test is also recommended for assessing corrosivity.[85–87]

Another model is the SkinEthic reconstituted human epidermal (RHE) model. There was good concordance between the *in vitro* predictions of skin corrosivity potential obtained with the SkinEthic model and the predictions obtained with other *in vitro* methods accepted by the OECD. The new test was able to distinguish between corrosive and noncorrosive reference chemicals with an accuracy of 93%.[88]

EPISKIN™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use in skin corrosivity testing involves applying test materials topically to the skin for 3, 60, and 240 min. The subsequent assessment of their effects on cell viability is measured by the MTT assay. Correlation between *in vitro* and *in vivo* data is high. The test was able to correctly identify corrosive and noncorrosive chemicals.[89–91] The Skin2™ model *in vitro* skin corrosivity test uses a three-dimensional human skin which has dermal, epidermal and corneal layers. The test is based on topically applying test materials to the stratum corneum of these human skin cultures. Subsequently, the effects on cell viability are assessed by the MTT assay.[92]

All of these skin models reproduce many of the characteristics of normal human epidermis. Therefore, they provide a morphologically relevant *in vitro* means of assessing skin irritation and of performing other skin-related studies.

The Transcutaneous Electrical Resistance Assay (TER)

This test assesses the skin corrosivity potential of a test material which is topically applied to the epidermal surfaces of skin discs. Such discs are obtained from humanely killed young rats. Corrosive substances produce an irreversible loss of normal stratum corneum integrity and function. This is measured as a reduction in the inherent transcutaneous electrical resistance (TER) below a corrosive threshold level.[93,94] The TER is a recommended regulatory approved screening test. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, measured as a reduction in the inherent TER below a predetermined threshold level (5 kohm). Nonirritant substances do not reduce the TER below the threshold level. The rat skin TER test proved that it could be applied to testing a diverse group of chemicals with different physical forms, including: organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols, and soaps/surfactants. There was good concordance between the skin corrosivity classifications derived from the *in vitro* and *in vivo* data. The test was able to distinguish between corrosive and noncorrosive chemicals for all of the chemical types studied. The rat skin TER test is scientifically validated for use as a replacement of the animal test for distinguishing between corrosive and noncorrosive chemicals. This test is accepted for regulatory assessment. The method has also been accepted by regulatory authorities. Since 2004, it has appeared in the OECD Guidelines as number 430.

ALTERNATIVES TO THE PHOTOTOXICITY TEST

Phototoxicity is defined as a toxic response that is elicited when skin is first exposed to certain chemicals and then exposed to light. Similarly,

it can be induced by skin irradiation after the systemic administration of a chemical.

One of the possible alternative test combines the haemolysis assay and the irradiation of RBCs with UV light. Such a method has two endpoints: photohaemolysis and methaemoglobin formation. These endpoints are determined by measuring changes in the optical density of the haemoglobin spectrum at 525 and 630 nm, respectively. In addition, a prediction model is inserted with two cut-off values: a photohaemolysis factor greater than or equal to 3.0 for photohaemolysis; and a delta OD greater than or equal to 0.05 for methaemoglobin formation. This method could be considered an *in vitro* test. It can be used advantageously to obtain some mechanistic information, in particular information on the photodynamic effects of a substance on cellular proteins and biomembranes.[95]

The RBC photohaemolysis assay is the most effective tool for assessing the phototoxicity of cosmetic ingredients.[96]

The 3T3 NRU phototoxicity assay is designed to detect the phototoxic potential of a chemical. It uses an *in vitro* cytotoxicity assay, involving the Balb/c 3T3 mouse fibroblast cell line. The basis of this test is a comparison of the cytotoxicity of the chemical when tested with and without exposure to a noncytotoxic dose of UVA light. Cytotoxicity is expressed as a concentration-dependant reduction in the uptake of the vital dye, neutral red, 1 day after treatment.[97,98]

The *in vitro* 3T3 NRU phototoxicity test is used to identify the phototoxic effect of a test substance induced by the combined action of a chemical and light. The test can identify substances that are phototoxic *in vivo* after being systemically applied and distributed to the skin. It can also identify compounds that could act as phototoxicants after topical application to the skin. The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test has been evaluated. It has been compared with acute phototoxicity effects *in vivo* in animals and humans, and has been shown to be predictive of these effects.[99]

The method has been validated and accepted and is recorded in the OECD Guidelines as Test 432.

Another possible endpoint for cytotoxicity is determined by the mitochondrial dehydrogenase conversion of a tetrazolium salt (MTT) to a coloured formazan product.[100–102]

Keratinocytes could also be used to identify photoirritant products.[62]

An additional proposed *in vitro* model is based on the use of two three-dimensional models: a dermal equivalent and a skin equivalent model. The dermal equivalent model includes a collagen-glycosaminoglycans-chitosan porous matrix populated by normal human fibroblasts. The skin equivalent model is made by seeding normal human keratinocytes onto the dermal equivalent. This leads to a fully differentiated epidermis.[103]

CONCLUSIONS

A large number of studies have been undertaken to find tests that replace the need for animals in eye and skin safety testing. However, few of these tests have been accepted by the regulatory authorities. At present, with the exception of corrosion and phototoxicity, no alternative *in vitro* tests for both ocular and skin irritation are available for regulatory purposes. Nevertheless many *in vitro* methods are applied in-house for laboratories around the world.

The application of tissue culture techniques, cellular and molecular biology, and analytic cytometric techniques can lead us closer to our goal of eliminating the need for animals in eye and skin irritation testing.

A battery of tests should be established, as no single assay can fulfil the requirements for risk assessment using an *in vitro* method.

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